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CARB-17 Family of β-Lactamases Mediates Intrinsic Resistance to Penicillins in *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus **is commonly resistant to ampicillin, yet the mechanisms underlying this phenomenon are not clear. In this study, a novel class A carbenicillin-hydrolyzing β-lactamase (CARB) family of β-lactamases,** ***bla*$_{CARB-17}$****, was identified and found to be responsible for the intrinsic penicillin resistance in** ***V. parahaemolyticus*****. Importantly,** ***bla*$_{CARB-17}$****-like genes were present in all 293** ***V. parahaemolyticus*** **genome sequences available in GenBank and detectable in all 91** ***V. parahaemolyticus*** **food isolates, further confirming the intrinsic nature of this gene.**

Vibrio parahaemolyticus is a major causative agent of gastroenteritis in areas with high seafood consumption rates and has recently become pandemic due to the emergence of the serotype O3:K6 (1). In Hong Kong, *V. parahaemolyticus* is the leading cause of food-borne illnesses due to the high rate of seafood consumption among the population (2). Although most cases of infections are self-limiting, fatality can occur among immunocompromised patients or those with debilitating medical conditions such as liver disease or diabetes (3). Antibiotics such as ciprofloxacin can be used for the treatment of infections caused by *V. parahaemolyticus* strains, but the choice of antibiotics should be based on the antimicrobial susceptibilities of the organism. *V. parahaemolyticus* is commonly considered highly susceptible to virtually all antimicrobials except for penicillins. However, mechanisms mediating the development of penicillin resistance in *V. parahaemolyticus* are not clear. In this study, we identified a novel carbenicillin-hydrolyzing β-lactamase (CARB) from chromosome 2 of *V. parahaemolyticus* and showed that the product of this gene is responsible for the intrinsic resistance to penicillins in *V. parahaemolyticus*.

A novel potential β-lactamase gene, *bla*$_{V110}$, with a length of 852 bp was identified through bioinformatics analysis of the whole-genome sequence of *V. parahaemolyticus* V110, which was shown to be resistant to ampicillin (4). The full-length novel β-lactamase gene was amplified by PCR using primer set F-GCT GAGAGCTCATGAAAAAGTTA, R-CGTAGGATCCTTAACTT TCTTTGTAGTGC and then cloned into *Escherichia coli* BL21 and tested for the MICs of various β-lactams according to CLSI standards (5). *E. coli* BL21 isolates expressing the novel β-lactamase gene exhibited MICs of 256, 512, 256, and 1,024 μg/ml toward penicillin G, ampicillin, carbenicillin, and piperacillin, respectively. The Bla$_{V110}$ enzyme appears to be susceptible to β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Table 1). To verify whether the penicillin resistance phenotype was attributed to the expression of *bla*$_{V110}$, we further purified a truncated form (60 to 852 bp) of this β-lactamase in which the signal peptide was removed and designated mBla$_{V110}$. The mBla$_{V110}$ protein was purified through several steps, including a Ni-nitrilotriacetic acid (NTA) column, thrombin treatment to remove the His tag, and a size exclusion column as previously described (6) (see Fig. S1 in the supplemental material). The purity of this protein was higher than 99%, and the yield of the purified mBla$_{V110}$ was about 2.4 mg/liter. Kinetic constants were determined for mBla$_{V110}$ as previously described (7), and very high catalytic activities on ampicillin, penicillin G, carbenicillin, and piperacillin were noted; however, this enzyme exhibited extremely low activities to other β-lactams tested (Tables 2). In general, mBla$_{V110}$ exhibited similar K_m values with all penicillins,

TABLE 1 MICs of different β-lactams on *V. parahaemolyticus* V110 parental strain and *E. coli* carrying pET28-*bla*$_{V110}$

Antibiotic[a]	MIC (μg/ml) against bacterial strain		
	V. parahaemolyticus V110	*E. coli* pET28-*bla*$_{V110}$	*E. coli* pET-28
Penicillin G	512	256	<1
Ampicillin	128	512	1
AMP/CLA (2:1)	2	<1	<1
AMP/SUL (1:1)	<1	4	<1
Carbenicillin	256	256	2
Piperacillin	256	1,024	<1
PIP/TAZ (10:1)	0.06	1	0.5
Cephalothin	8	1	0.03
Cefuroxime	8	0.25	0.03
Cefotaxime	0.06	0.004	0.004
Cefepime	1	0.06	0.06
Cefpirome	0.25	0.015	0.015
Aztreonam	4	0.008	0.004
Imipenem	0.03	0.25	0.008

[a] AMP, ampicillin; CLA, clavulanic acid; SUL, sulbactam; PIP, piperacillin; TAZ, tazobactam.

Received 8 January 2015 **Returned for modification** 4 February 2015
Accepted 15 March 2015

Accepted manuscript posted online 23 March 2015

Citation Chiou J, Li R, Chen S. 2015. CARB-17 family of β-lactamases mediates intrinsic resistance to penicillins in *Vibrio parahaemolyticus*. Antimicrob Agents Chemother 59:3593–3595. doi:10.1128/AAC.00047-15.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00047-15.

doi:10.1128/AAC.00047-15

TABLE 2 Kinetic constants of $mBla_{V110}$ toward different β-lactams

Antibiotic	$mBla_{V110}$		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)
Penicillin G	110.4 ± 19.31	2,320 ± 189.2	21.01
Ampicillin	235.8 ± 40.44	2,068 ± 170.9	8.77
Carbenicillin	113.9 ± 28.61	1,233 ± 177.4	10.83
Piperacillin	32.6 ± 8.87	450.7 ± 50.73	13.82
Cefuroxime	NH[a]	<0.01	
Cefotaxime	NH	<0.01	
Cefepime	104.3 ± 25.67	0.74 ± 0.08	7.10×10^{-3}
Cefpirome	22.6 ± 4.77	0.98 ± 0.07	4.34×10^{-2}
Aztreonam	NH	<0.01	
Imipenem	NH	<0.01	

[a] NH, no detectable hydrolysis was observed with 1 μM purified $mBla_{V110}$ and up to 500 μM substrate.

cefepime, and cefpirome tested but variable k_{cat} values to these penicillins, some cephalosporins, aztreonam, and imipenem (Tables 2). The kinetic data were highly consistent with the MICs of *E. coli* isolates carrying bla_{V110} genes. Collectively, our data suggested that Bla_{V110} is an active β-lactamase that mediates the resistance to penicillins in the *V. parahaemolyticus* V110 strain.

Protein BLAST of Bla_{V110} showed 99% homology with PSE-4 from *V. parahaemolyticus* (8, 9) and high homology (54%) with PSE-4 from *Pseudomonas* spp. PSE-4 is an alternative name for CARB-1, which belongs to the CARB-type family originally identified from *Pseudomonas aeruginosa*, *Acinetobacter*, and *Vibrio cholerae* (10–12). CARBs, also known as carbenicillin-hydrolyzing β-lactamases, have been found to disperse widely among distantly related bacteria, mostly by mobile genetic elements (10, 13, 14). Similarly, Bla_{V110} also mediated resistance to ampicillin, penicillin G, carbenicillin, and piperacillin. Therefore, Bla_{V110} was designated a novel member of the CARB family, $bla_{CARB-17}$ (GenBank accession number KJ934265).

Analysis of its genetic environment showed that the $bla_{CARB-17}$ gene was located on chromosome 2 of *V. parahaemolyticus* V110. Several putative genes including those encoding transporters and enzymes are located upstream and downstream of $bla_{CARB-17}$ (see Fig. S2 in the supplemental material). The $bla_{CARB-17}$-like genes were also identified in chromosome 2 of 5 *V. parahaemolyticus* isolates with completed whole-genome sequences in GenBank (see Fig. S2). The genetic environments of the $bla_{CARB-17}$-like genes in these isolates were very similar but not identical. There were no mobile genetic elements, such as integrase and transposase, within their genetic environments, and the $bla_{CARB-17}$-like genes were not located in any genomic islands, suggesting that $bla_{CARB-17}$ genes may be intrinsic to *V. parahaemolyticus*. Further analyses of the other 292 whole-genome annotation reports of *V. parahaemolyticus* available in GenBank identified $bla_{CARB-17}$-like β-lactamases in 280 out of the 292 *V. parahaemolyticus* strains. $bla_{CARB-17}$-like β-lactamase genes were also identified in the other 12 *V. parahaemolyticus* strains, but they either showed one nucleotide deletion within the full-length $bla_{CARB-17}$-like genes (10 strains) or showed longer nucleotide fragment deletion at the N termini (2 strains). The latter two strains have very low numbers of proteins in their annotation reports, implying that the lack of full-length $bla_{CARB-17}$-like genes may be due to the sequencing coverage issue. Taken together, complete genome analysis suggested that all *V. parahaemolyticus* strains intrinsically harbor $bla_{CARB-17}$ and its variants. To further prove the intrinsic nature of $bla_{CARB-17}$, 39 and 52 *V. parahaemolyticus* strains isolated from seafood in Shenzhen and Hong Kong, respectively, were screened for the presence of $bla_{CARB-17}$-like genes using primers targeting the full length of $bla_{CARB-17}$. These isolates were confirmed to be *V. parahaemolyticus* through screening for the presence of *tlh* and *atpA* genes as well as API20E assays (bioMérieux). All isolates were resistant to ampicillin with the exception of 10 isolates with ampicillin MICs of 16 μg/ml. All strains showed positive amplifications for $bla_{CARB-17}$, and these genes were further confirmed to be $bla_{CARB-17}$-like genes by sequencing. These data further confirmed the intrinsic nature of $bla_{CARB-17}$-like genes in *V. parahaemolyticus*.

The current nomenclature for the CARB family of β-lactamases is confused with the PSE family in the literature. CARBs are divided into two subgroups, namely the CARB and RTG subgroups (http://www.lahey.org/Studies/). Based on the functional characterization of novel CARB-17 and its variants, the CARB family of β-lactamases can be separated into three distinct squares through further phylogenetic analyses of all CARB β-lactamases. The first square contains CARB-17 and its closely related variants, the second square contains mainly the previously identified narrow-spectrum CARB family from *Pseudomonas* spp. and *V. cholerae*, and the third square contains the broad-spectrum RTG subgroup (Fig. 1).

In conclusion, this study identified a novel class A β-lactamase, $bla_{CARB-17}$, which is responsible for the intrinsic resistance to penicillins in *V. parahaemolyticus*. The data facilitate clear grouping for the whole family of the CARB class of β-lactamases.

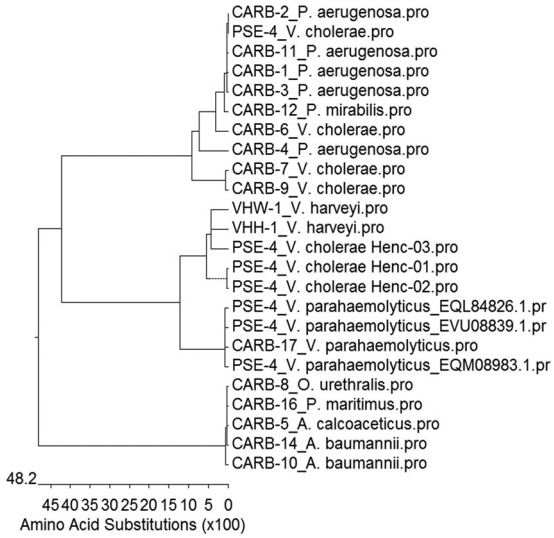

FIG 1 Phylogenetic tree of CARB family and several related β-lactamases. CARB subgroups, including CARB-1 (PSE-4), CARB-2 (PSE-1), CARB-3, CARB-4, CARB-6, CARB-7, CARB-9, and CARB-11 (PSE-5) to CARB-15, are narrow-spectrum β-lactamases that hydrolyze penicillins (15, 16). RTG subgroups, including CARB-5 (RTG-2), CARB-8 (RTG-3), and CARB-10 (RTG-4), consist of an RTG triad as reported for the GN79 (RTG-1) from *Proteus mirabilis* (11, 14, 17, 18). Most of the CARB families were not subjected to functional characterization except CARB-10, which has been shown to hydrolyze cefepime and cefpirome and become an extended-spectrum CARB enzyme (14). CARB-17 exhibited 99% homology to PSE-4 from *V. parahaemolyticus* and approximately 80% to PSE-4 from *V. cholerae* Henc, as well as VHW-1 and VHH-1 from *Vibrio harveyi* through BLAST analysis (19). The latter two β-lactamases have been shown to be active on penicillins (19).

ACKNOWLEDGMENTS

We thank Kathy Po for providing the *V. parahaemolyticus* isolates for $bla_{CARB-17}$ gene screening, Yuqian Wu for his help with $bla_{CARB-17}$ cloning and expression, and Edward Chan for critical reading of the manuscript.

This work was supported by the Chinese National Key Basic Research and Development (973) Program (grant 2013CB127200) and the Health and Medical Research Fund from the Food and Health Bureau, Government of the Hong Kong SAR (grant HMRF:13121422 to S.C.).

We declare that we have no conflicts of interest.

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